Intraperitoneal administration of high doses of polyethylene glycol (PEG) causes hepatic subcapsular necrosis and low-grade peritonitis with a rise in hepatic biomarkers

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Liverpool, 07-Apr-2013

Dear Editor-In- Chief,

I am sending you our manuscript entitled "Intraperitoneal administration of high doses of polyethylene glycol (PEG) causes hepatic subcapsular necrosis and low-grade peritonitis with a rise in hepatic biomarkers" by Giovanni Pellegrini et al. I would appreciate if you could consider it for publication as a "Short Communication" type of manuscript in "Toxicology".

To our knowledge, this is the first report showing liver necrosis and generalised peritonitis in mice receiving PEG-400, a vehicle commonly used as excipient in preclinical and *in vitro* studies. This work is the result of a collaboration between two Departments at the University of Liverpool (Pharmacology and Veterinary Pathology), which are both part of our MRC Centre for Drug Safety Science, directed by Professor Kevin Park.

We thought that our findings would appeal to the readership of Toxicology, as they are new and concern a commonly used chemical entity. As such, I would like to confirm that this manuscript is not currently under consideration by another journal. In addition, all authors have approved the manuscript, agree with its submission to Toxicology and declare that there are no conflicts of interest.

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We look forward to hearing from you at your earliest convenience.

Yours' faithfully,

Giovanni Pellegrini

Intraperitoneal Administration of High Doses of Polyethylene Glycol (PEG) Causes Hepatic Subcapsular Necrosis and Low-Grade Peritonitis with a Rise in Hepatic Biomarkers

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ABSTRACT: Polyethylene glycols (PEGs) are commonly employed as excipients in preclinical studies and *in vitro* experiments to dissolve poorly hydrosoluble drugs. Their use is generally considered safe in both animals and humans; however, limited data is available concerning the safety of PEGs when administered parenterally. The results of our investigation demonstrate that PEG-400 can have an irritant effect on serosal surfaces and causes subcapsular hepatocellular necrosis in mice when administered intraperitoneally at a high dose. Accordingly, levels of serum biomarkers of liver injury need to be carefully interpreted in studies where PEG is administered intraperitoneally and always in association with the results of the histological assessment.

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Keywords: polyethylene glycol; excipients; hepatic biomarkers; hepatic subcapsular necrosis; peritonitis.

1. INTRODUCTION

Polyethylene glycols (PEGs) comprise a class of synthetic polymers composed of identical ethylene oxide subunits, with molecular weights varying between 200 and 10,000. PEGs are known for their versatile range of industrial applications and are ideal candidates for the development of solvents, vehicles and emulsifying agents in the pharmaceutical and cosmetic industries (Fruijtier-Pölloth, 2005). In addition, PEGylation of therapeutic products has become in the last two decades an attractive and effective tool to modulate the pharmacological properties of drugs, such as solubility, stability and immunogenicity, and to improve their metabolic and toxicological profiles (Pasut et Veronese, 2011).

Due to their extensive use and the potential chronic exposure of humans through different routes, the acute and repeat dose toxicity of PEGs has been widely investigated in several preclinical species. PEGs and pegylated products generally have excellent safety profiles, with toxicity occurring only with high parenteral doses that far exceed the concentrations normally used in practice (Fruijtier-Pölloth, 2005).

Due to the strong hydrophilic properties, low molecular weight PEGs, such as PEG-400, have been used in non-clinical studies to deliver poorly hydrosoluble drugs by different routes, including intraperitoneal (ip) administration (Gad et al., 2006; Rowe et al., 2009).Limited data concerning dosing regimens and safety of PEGs injected directly into the abdominal cavity are available, as this route of administration is not commonly used in humans. Studies investigating the acute ip toxicity of low molecular weight PEGs in mice determined a LD50 of 9.2 g/kg and a NOEL (“no observed effect level”) of 1 ml/kg (Shideman et Procita, 1951; Worthley et Schott, 1966; Rowe et al., 2009). No assumption has been made regarding the cause of death of mice administered an ip lethal dose of PEG, but it is likely this could be attributed to a rapidly developing hypovolaemic shock. This assumption is supported by the fact that, given ip at high doses (between 3.5 g/kg and 8 g/kg), PEG induces a reduction in plasma volume without altering plasma osmolality. Accordingly, due to its osmotic activity,

it has been used in an experimental model of hypovolaemia in rats and mice (Fitzsimons, 1961; Dunn et al., 1973; Hashimoto et al., 2010).

At our facilities, PEG-400 has been employed as a suitable ip vehicle in a murine model of hepatotoxicity induced by furosemide (FS) (Williams et al., 2007). As anticipated, FS caused extensive centrilobular hepatocellular necrosis, associated with substantial increases in alanine aminotransferase (ALT) serum activity and raised levels of circulating micro-RNA (MiR-122); however, we also observed biomarker levels above normal historical background ranges in several vehicle-control (PEG-400) mice. Furthermore, a few vehicle-control mice also exhibited multifocal subcapsular hepatic necrosis. These changes complicated the interpretation of drug-related biomarker increases and prompted an investigation to elucidate the relationship between the observed changes and PEG-400 application.

2. METHODS

2.1 Study animals and procedures

The protocols described were undertaken in accordance with criteria outlined in a license granted under the Animals (Scientific Procedures) Act 1986 and approved by the University of Liverpool Animal Ethics Committee. All animals were obtained from Charles River (Margate, UK).

Retrospective analysis of the controls from furosemide toxicity studies at our facility identified 60 male and female CD-1 and C57BL6 mice (5-7 weeks old, 25–35 g at study start) that had received a single ip injection of PEG-400 (density: 1.126 g/mL) at 4 mL/kg of body weight. Animals had been killed with a raising concentration of carbon dioxide, followed by exsanguination by cardiac puncture at 1, 5, 8 or 24 h post treatment. A complete gross post mortem examination had been performed on each mouse, gross abnormalities recorded and all tissues collected in 10% non-buffered formalin for histological analysis. Further studies using the same conditions and procedures described earlier were conducted on 15 male CD-1 mice to investigate the effect of different PEG-400 formulations (Sigma-Aldrich and Fischer) and the effect of PEG-400 at early time points (1, 3 and 5 h) post ip administration. The pH of the formulations was checked to be between 7.3 and 7.6 prior to administration. The formulations were certified for purity and identity by the manufacturers, which guarantee that the levels of residual solvents, heavy metals and reducing substances are within the limits of acceptance. From selected

mice euthanased 1, 3 and 5 h after treatment, portions of subcapsular hepatic parenchyma from different lobes were rapidly removed and fixed in 2.5% gluteraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for transmission electron microscopy (TEM).

2.2 Determination of serum alanine transaminase activity and circulating MiR-12

Blood samples were stored at 4°C and allowed to clot overnight. Serum was obtained from separation by pulse centrifugation (4000 rpm). Alanine transaminase activity (ALT) was determined in all animals using ThermoTrace Infinity ALT Liquid stable reagent according to the manufacturer's instructions. In 12 male CD-1 mice euthanased at 5 and 8 h after PEG administration, miRNA levels were measured using a Taqman-based quantitative polymerase chain reaction (PCR), as previously described (Antoine et al., 2009).

2.3 Histological, immunohistological and ultrastructural examination

From all animals, multiple sections from the left and median hepatic lobes were prepared from formalin-fixed livers. From the 15 animals euthanased at 1, 3, 5 and 8 hours post treatment, sections of the gastrointestinal tract (glandular and non glandular stomachs, duodenum, jejunum, ileum, caecum and colon), mesenteric lymph node, mesentery, spleen, kidneys, diaphragm, pancreas and the injection site (ventral abdominal muscle wall) were also formalin-fixed and processed. After routine paraffin wax embedding, sections (3-5 µm) were prepared and stained with haematoxylin and eosin. Selected liver sections were also stained with a Martius Scarlett Blue stain for the detection of fibrin and/or the Masson trichrome stain to highlight the connective tissue of the Glisson's capsule.

The extent of hepatocellular subcapsular necrosis was graded (grade 0-4), with 0: no changes or a negligible proportion of parenchyma affected; 1: focal or patchy single layer-deep subcapsular hepatocyte necrosis with occasional neutrophils; 2: multifocal, one or two layer-deep hepatocyte necrosis, surrounded by a thin continuous rim of neutrophils; 3: multifocal or diffuse, several layer-deep subcapsular hepatocyte necrosis with marked neutrophil infiltration; 4: diffuse subcapsular coagulative hepatocyte necrosis extending deep into the liver parenchyma, accompanied by marked neutrophil infiltration.

Immunohistology for the demonstration of TNF-α, IL-6, cleaved caspase 3 and proliferating cell nuclear antigen (PCNA) was performed on selected CD-1 mice euthanased at 3, 5, 8 and/or 24 h after dosing, following previously described protocols (Antoine et al., 2009).

For TEM, fixed liver sections of selected CD-1 mice euthanased at 1, 3 and 5 h after dosing were routinely epoxy resin embedded. 3 µm toluidine blue stained semithin sections were used to select representative areas for 75 nm ultrathin sections, which were contrasted with uranyl acetate and lead citrate.

2.4 Assessment of PEG-induced cytotoxicity in primary murine and human hepatocytes.

Human Hep G2 and murine Hepa1c1c7 cells were used as test cell lines to assess the cytotoxicity of PEG-400 at varying concentrations. Both cell lines were maintained in Dulbecco's Modified Eagles' Medium (DMEM) containing glucose, L-glutamine, sodium pyruvate and sodium bicarbonate, supplemented with 10% foetal bovine serum and 1% penicillin streptomycin in T75 cell culture flasks and incubated for a minimum of 18 h in 96 well plates prior to incubation with PEG-400. Cells were dosed with PEG-400 in eight different concentrations, ranging from 0 to 750 µM (0-30% concentration). Three wells per plate were incubated with 250 µM of the antidepressant nefazodone, a known hepatotoxin, as positive controls. Plates were incubated for a period of 3, 5 or 24 h and subsequently tested for cell viability by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) and ATP assays, according to the manufacturer's instructions.

3. RESULTS

3.1 In vivo studies

ALT levels were above the reference intervals cited in the literature (Loeb et Quimby, 1999) in 45% of mice receiving PEG-400, regardless of sex and strain. Biomarker increases occurred in mice euthanased at 5 and, more consistently, 8 h after dosing, where almost half of the cohort had serum levels ranging from just above the mean reference to 8 fold increases (Figure 1A). Circulating miR-122 levels were determined in 12 examined mice. These were within our historical background reference value (1.0 ΔΔCt miR-122/lin-4) in mice at 5 h after dosing. However, at 8 h, two of the six mice

examined exhibited increased levels above this limit. These were associated with increased ALT serum levels (Figure 1B).

Post mortem examinations revealed ascites with up to 600 µl of clear transparent fluid in mice euthanased at 1 or 3 h after dosing, against a maximum volume of 200 µl of PEG-400 administered into the abdominal cavity. No other macroscopical changes were detected.

The main histological finding was hepatocellular subcapsular necrosis. This was observed to a variably degree in 63% (n=47) of the mice (Figure 2). Necrosis was detected at all time points after 1 h post dosing, with an increase in incidence and severity from 5 to 8 h time points, while no appreciable difference was noted between the 8 and 24 h cohorts (Table 1). Histopathological scores were not influenced by gender, mouse strain or the formulation used.

The subcapsular hepatocellular necrosis was focal to multifocal, patchy or extensive (involving almost the entire liver section). It affected one to several hepatocyte layers beneath the Glisson's capsule. Necrotic hepatocytes were lined by scattered to large numbers of degenerate (karyorrhectic) neutrophils, accompanied by extravasated erythrocytes. Above subcapsular necrotic areas, small amounts of fibrin (Martius Scarlett Blue stain: positive) were occasionally found attached to the serosa, mesothelial cells were not readily identifiable and the Masson trichrome stain showed that the capsular connective tissue layer in these areas was disorganised and split into irregularly arranged discontinuous fibres (Figure 2E).

The immunohistological examination of CD-1 mice euthanased at 3 h after dosing showed that neutrophils infiltrating the areas of hepatocyte necrosis expressed both the proinflammatory cytokines TNF-α and IL-6. At 24 h post dosing, increased numbers of PCNA-positive, proliferating hepatocytes were present in the periphery of the necrotic areas, indicating the onset of hepatocyte regeneration (Figure S1, supplementary data). The lack of cleaved caspase 3 expression in degenerate hepatocytes confirmed necrosis as the mode of hepatocyte death (data not shown).

The ultrastructural examination confirmed both the loss of mesothelial cells and acute subcapsular hepatocyte necrosis in affected areas (Figure 3).

In 40% (6/15) of the mice where abdominal organs were examined in addition to the liver, slight to mild neutrophilic infiltration was observed beneath the serosal layer of the intestine (especially the ileum),

the diaphragm and the abdominal wall (Figure S2, supplementary data). This was occasionally associated with mild focal haemorrhage and scattered myofibre degeneration in the latter.

3.2 In vitro studies

In order to corroborate our *in vivo* observations, human and murine hepatoma cell lines were exposed for 5, 8 and 24 h to gradual concentrations (0 to 30%) of PEG-400, diluted in isotonic saline. A dose-dependent reduction of cell viability was detected by (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) and ATP assays in both cell lines with both assays even at the lowest dilution tested (2.5%), confirming the cytotoxic effect of PEG (Figure S3, supplementary data).

DISCUSSION

The present study reveals the cytotoxicity of PEG-400 at high concentrations. When administered ip at a high dose to mice without prior dilution, PEG-400 has an apparent cytopathic effect on mesothelial cells and hepatocytes immediately beneath the serosal surface, leading to necrosis and a neutrophilic inflammatory response. *In vivo* data are paralleled by the evidence of hepatocytotoxicity exerted by PEG-400 on permanent cell lines derived from human and murine neoplastic hepatocytes, even at very low concentrations.

Data concerning the safety of parenteral administration of PEG products are scant. A maximum dilution of 30% is recommended when PEG is administered intravenously to humans and dogs, as higher concentrations may cause haemolysis (Rowe et al., 2009; Li et al., 2011). It remains to be clarified whether this is also a consequence of the hydrophilic properties of PEG-400 which are in our study the apparent cause for the local recruitment of fluid into the peritoneal cavity early (1 and 3 h) after its ip administration. However, it may be hypothesised that PEG-400 exerts a local cytotoxic effect on mesothelial cells and hepatocytes within the first minutes after administration, prior to dilution of the inoculum by the fluid recruited into the peritoneal cavity.

This study provides a preliminary data for preclinical toxicological studies using PEG-400 as a vehicle for intraperitoneal administration. However, further investigation is needed to clarify whether the low PEG-400 concentrations seen to reduce cell viability *in vitro* are able to cause clinical and pathological

findings analogous to those observed *in vivo* with ip administration of undiluted PEG and to compare the safety of PEG with various molecular weights. Recent research on hepatotoxicity has focused on the development of new reliable and specific biomarkers of hepatic injury, which should be able to sensitively predict toxicity in preclinical models. PEG injury could lead to enhanced damage when administered with test compounds, potentially complicating a mechanistic interpretation. This current work underscores the critical role of histopathology as a compulsory tool for the correct interpretation of biomarker level variations and stresses once again the importance of research into new, mechanism-specific biomarkers of hepatocellular injury.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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Figure and Table legends

Figure 1. A. Box-plots for ALT levels measured in the plasma obtained at terminal bleeding of mice at different time points after ip PEG-400 (4 mL/kg) administration. Numbers of mice evaluated in each cohort are displayed in brackets on the legend of the x axis. Values on the ordinate are the natural logarithm of the actual ALT measurement Dotted lines represent mean ALT reference values for male (top line, 120 U/L, SD: 106.7 U/L) and female (bottom line, 62 U/L, SD: 54.8 U/L) CD-1 mice. B. Correlation between ALT and circulating miR-122 levels. miRNA levels were determined in 12 CD-1 mice administered PEG-400 (4 mL/kg) ip and euthanased 5 or 8 h after dosing. Values on both axes are expressed as the base-10 logarithm of the actual measurements.

Figure 2. Histological changes in the liver of mice after ip administration of PEG-400 (4 mL/kg). A to C. Hepatic subcapsular necrosis (arrows) grade 1 (A, CD-1 mouse, 5 h post treatment), (B, C57/B6 mouse, 5 h post treatment) and grade 4 (C, C57/B6 mouse, 24 h post treatment), characterised by coagulative necrosis of hepatocytes, neutrophil infiltration and blood extravasation; haematoxylin and eosin stain, 20 x. D and E CD-1 mouse, 8 h post treatment. The Glisson's capsule represents one dense continuous line (arrow) in unaffected areas (D); in areas overlying a layer of hepatocyte necrosis (E) the capsule is disrupted and appears as a fragmented undulating blue line (arrowheads); Masson trichrome stain, 20 x.

Table 1. Incidence and severity of hepatic subcapsular necrosis in mice at different time points (1, 5, 8 and 24 h) after ip PEG-400 administration at a dose of 4 mL/kg, based on the histological evaluation of several liver sections of each animal.

Figure 3. Ultrastructural changes in the liver capsule and underlying parenchyma of a CD-1 mouse, 3 h post ip administration of PEG-400 (4 mL/kg) A) The hepatocytes immediately beneath the capsule are vacuolated or necrotic, whereas the underlying hepatocytes (below the dotted line) appear unaltered (arrowhead: nucleus of hepatocyte). Neutrophils (arrows) are present adjacent to damaged

hepatocytes. B) Higher magnification of the transition (dotted line) between a necrotic (top) and viable (bottom) hepatocyte. The hepatocyte undergoing necrosis exhibits dilated mitochondria (arrows) and in the nucleus (arrowhead) disintegration of the nuclear membrane and the nucleolus and clumping of chromatin. C) Mesothelial cells are lost (*) and the capsule is expanded and infiltrated by neutrophils (arrows). D) Unaffected area, with capsule covered by a viable mesothelial cell (arrow) with intact microvilli (arrowhead).

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Subcapsular necrosis	1 h	5 h	8 h	24 h
Grade 0	5	12	6	5
Grade 1	0	4	9	5
Grade 2	0	9	7	3
Grade 3	0	3	3	3
Grade 4	0	0	0	2
% affected mice	0/5	44 (16/28)	75 (19/24)	72 (13/18)
Average severity	0	1.11	1.21	1.56

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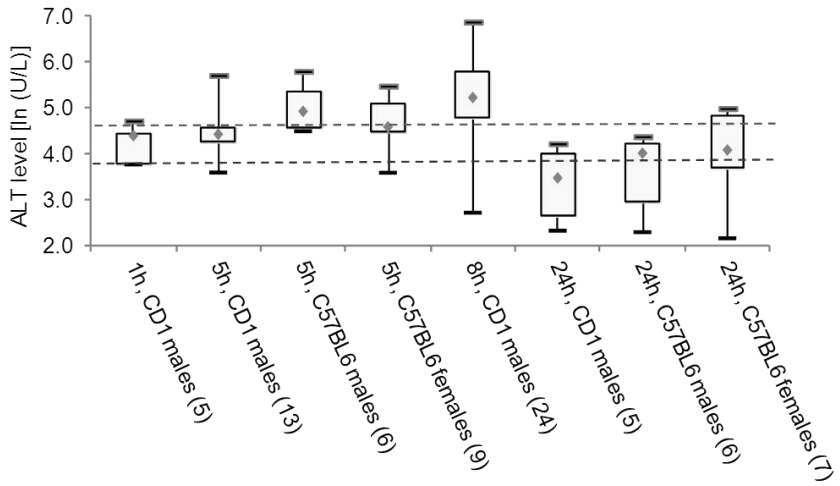

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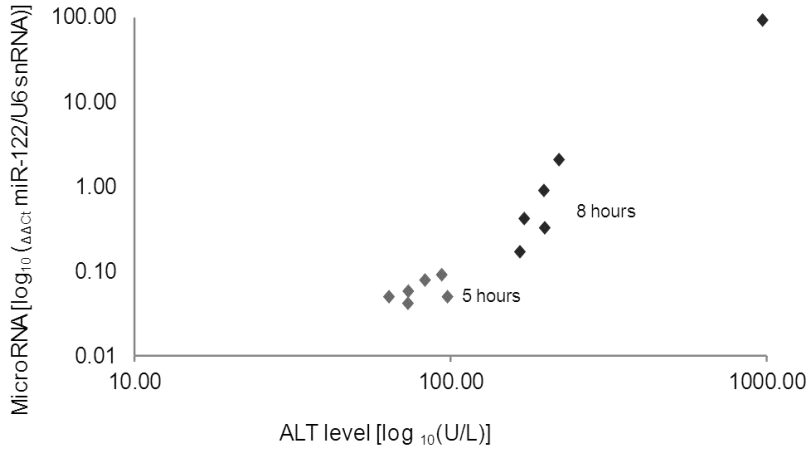

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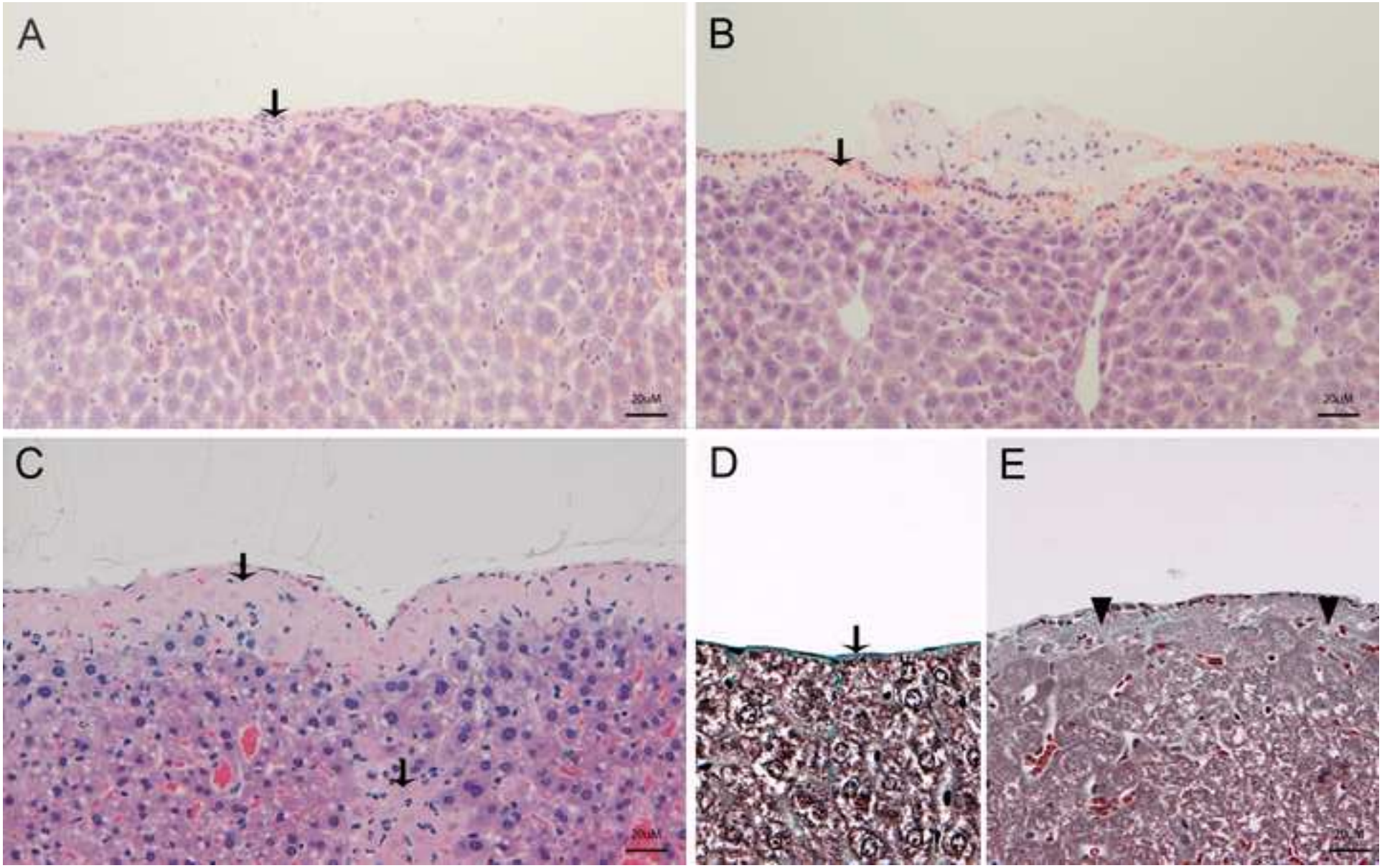

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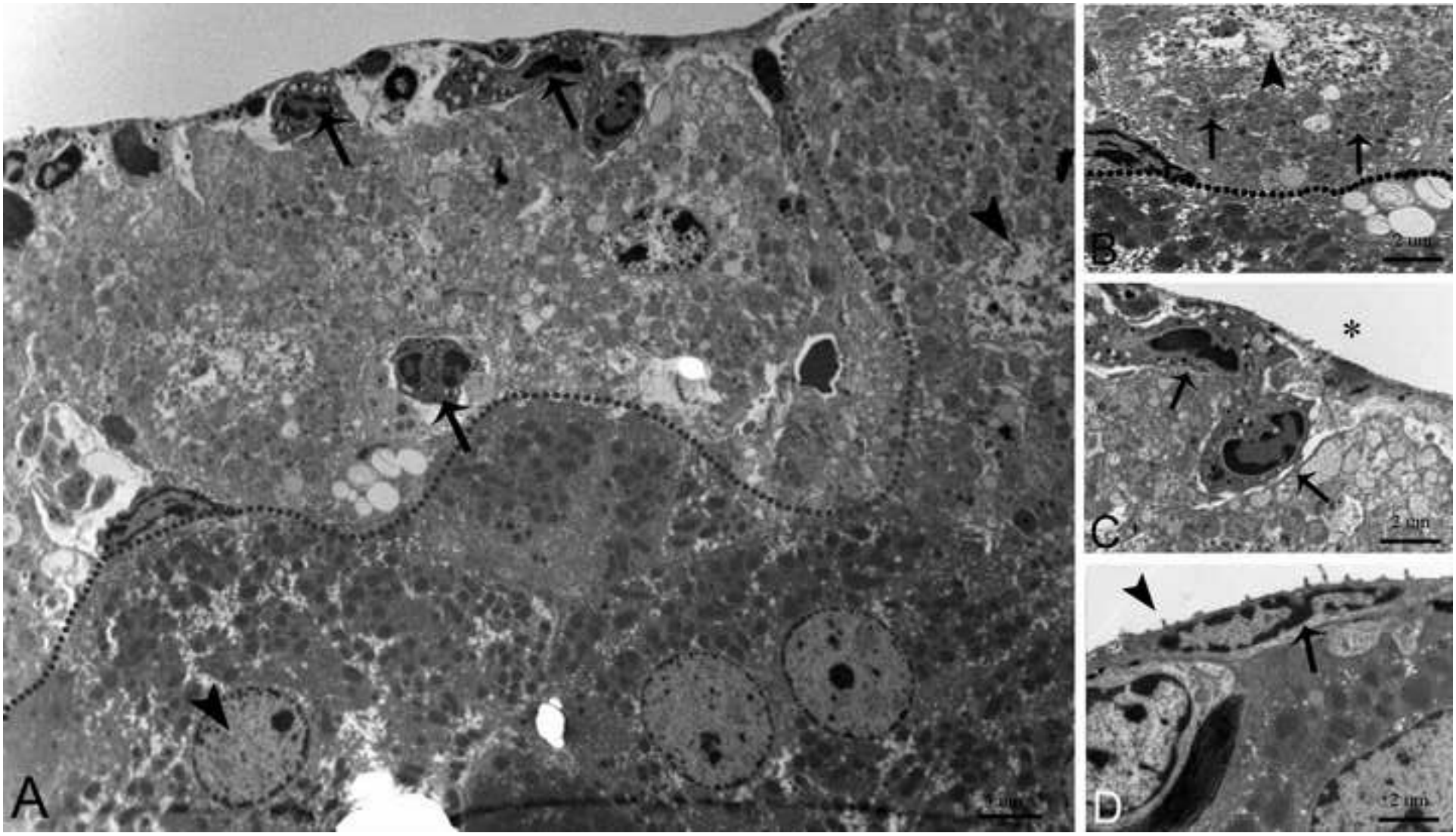

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Author name: Phil Starkey Lewis

Declarations

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All sources of funding should be acknowledged and you should declare any extra funding you have received for academic research of this work. If there are none state 'there are none'.

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Print name Phil Starkey Lewis

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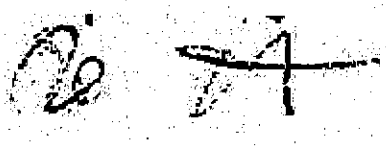

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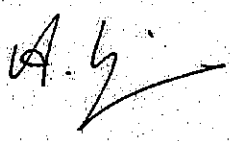
Print name Anja Kipar _____



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Toxicology

Conflict of Interest Policy

Article Title: Intraperitoneal Administration of High Doses of Polyethylene Glycol (PEG) Causes Hepatic Subcapsular Necrosis and Low-Grade Peritonitis with a Rise in Hepatic Biomarkers

Author name: Luke Palmer

Declarations

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Please declare any financial or personal interests that might be potentially viewed to influence the work presented. Interests could include consultancies, honoraria, patent ownership or other. If there are none state 'there are none'.

Please state any competing interests

There are none

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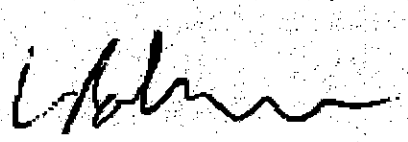

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Author name: Christopher Goldring

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Author name: Dominic Williams

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Article Title: Intraperitoneal Administration of High Doses of Polyethylene Glycol (PEG) Causes Hepatic Subcapsular Necrosis and Low-Grade Peritonitis with a Rise in Hepatic Biomarkers	Author name: GIOVANNI PELLEGRINI

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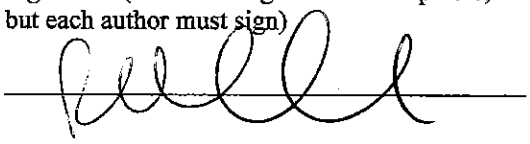

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GIOVANNI PELLEGRINI

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Article Title: Intraperitoneal Administration of High Doses of Polyethylene Glycol (PEG) Causes Hepatic Subcapsular Necrosis and Low-Grade Peritonitis with a Rise in Hepatic Biomarkers	Author name: KEVIN PARK

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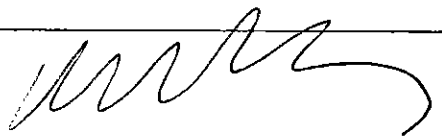

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KEVIN PARK